Detection of Thyroid-Stimulating Immunoglobulins by Use of Enzyme-Fragment Complementation

To the Editor:

In Graves disease, autoantibodies activate thyroid-stimulating hormone (TSH) receptor, leading to hyperthyroidism caused by increases in intracellular cAMP and thyroid hormone. We developed a nonradioactive assay to measure thyroid-stimulating immunoglobulins (TSIs).

Fasting serum samples were collected and deidentified for the reference interval study, which was approved by the University of Utah institutional review board (IRB #7740). Chinese hamster ovary–hTSHR-25–adherent cells (passage <30) (Leonard Kohn, Interthyr Research Foundation) were propagated in Ham F12-media supplemented with 5% serum and were trypsinized and dispensed into a 96-well plate at a density of 16 000 cells/well 48 h before use. The medium was removed from the cells, and 100 µL of Hanks balanced salt solution was added to each well. To predilute serum samples, 70 µL of serum was dispensed into 530 µL hypotonic Hanks Balanced Salt Solution in a 2-mL deep 96-well block. We then added 85 µL of diluted serum to each well of the cell culture plate. The final volume/well was 185 µL. All samples were tested in triplicate. Cells were incubated at 5% CO2, 37 °C for 90 min. After stimulation with sera, cAMP in the supernatant was measured using a cAMP-RIA reagent set (Amersham Biosciences). Lysis of the remaining monolayer and detection of intracellular cAMP concentrations were performed with the DiscoverRx HitHunter cAMP XS enzyme fragment complementation (EFC-cAMP) assay according to the manufacturer’s instructions. For a 96-well plate, we used 25 µL of cAMP XS antibody/lysis, 25 µL of enzyme donor–cAMP conjugate, and 50 µL of enzyme acceptor/chemiluminescence substrate. The cAMP calibrator was diluted in Hanks Balanced Salt Solution/3-isobutyl-1-methylxanthine mixture as described above, and 15 µL of the diluted calibrator was placed in a separate 96-well cell culture plate and treated identically to the cells. After substrate addition, the plate was incubated for 18 h at room temperature. We then transferred 60 µL to 1 white 384-well plate (Thermolabsystems) and read it on a luminometer (Thermolabsystems). All dispensing steps were performed using the Tecan Freedom Evo 200. Calibrators were plotted using a 4-parameter best-fit analysis. cAMP concentrations were measured on either the Immulite or Roche Modular E170. We performed data analyses by use of the online EP evaluator (David G. Rhoads; http://www.dgrhoads.com) and CBstat (Kristian Linnet). Indeterminate was defined as 110%–129% of the TSI reference interval value; a positive result was set at ≥130% and was consistent with Graves disease.

Results for the same stimulated cells processed with the RIA-cAMP and the EFC-cAMP methods correlated well [R = 0.96 (P < 0.001), n = 49, slope = 1.06, and intercept = −18.27 (Fig. 1)]. Two samples positive by RIA-cAMP were negative and indeterminate by EFC-cAMP [mean (SD) RIA-cAMP and EFC-cAMP values 156% (12.66%), 95% (2.9%); and 140% (6.0%), 119% (1.7%), respectively], and 1 sample was indeterminate by RIA-cAMP and positive by EFC-cAMP (111% (8.6%), 143% (11.2%)). An additional 376 samples assayed with the RIA-cAMP method were processed using the EFC-cAMP method. The correlation for all 425 samples was R = 0.92, slope 1.048, intercept = −2.2 by Deming regression. Use of the EFC-cAMP assay according to the traditional method may lead to false-positive results at TSH concentrations 10 mU/L. Such occurrences should not be a problem, however, because patients with Graves disease with hyperthyroidism should have TSH concentrations below the reference interval (<0.4 mU/L) (data not shown).

Results for 5 of 6 Graves patient samples provided by Kronus from patients clinically diagnosed with Graves disease were positive (TSH concentrations 370%, 382%, 371%, 262%, and 316%) and 1 was indeterminate (115% of the reference interval TSI value). For correlation, 3 samples were assayed both in our laboratory and a second reference laboratory: the results were 100%, 106%; 446%, 278%; and 149%, 180% of the TSI reference interval value; respectively. Results for 1 sample positive for EFC-cAMP were negative twice by RIA-cAMP (85%, 106%) and positive by TRAb (68% inhibition of TSH binding). Intraassay precision was tested by running 5 samples 3 to 4 times in triplicate, with an observed CV of 10%. During a 20-day period, with a mean sample result of 429%, the interassay CV was 27%. The interassay CV for a second control was 9.6%. Two cAMP controls, tested independent of the Chinese hamster ovary cells for 140 runs, had a CV of 15%.

Data regarding assay specificity (including the results for testing samples from patients with Hashimoto disease) and sensitivity for the cell line were previously published (1). Pooled normal-reference serum run 11 times in tripli-
cate yielded an SD of 5.5% of the reference interval value. In addition, 46 serum samples from healthy individuals were tested using the EFC-cAMP method (mean 94.4%, median 94.5%, and SD 6.8%). Results for the normal samples were 80% to 106% of the control (data not shown).

This EFC-cAMP assay is a nonradioactive surrogate for RIA-cAMP for studying antibody-mediated increases in cAMP concentrations in response to activation of the G-coupled TSH receptor.

Grant/Funding Support: This work was supported by the ARUP Institute for Clinical and Experimental Pathology.

Financial Disclosures: None declared.

Acknowledgments: We thank Leonard Kohn for providing the Chinese hamster ovary cell line expressing the TSH receptor (Leonard Kohn, The Interthyr Research Foundation), Linda Seiler for excellent technical expertise, Hayden Jeffreys for samples from Graves patients (Kronus), and Lois Hybl and Tom Martins for critical reading of the manuscript.

References

Tanya Sandrock1*
Alan Terry1
Jeff C. Martin1
Evrim Erdogan2
Wayne A. Meikle2,3

1 ARUP Institute for Clinical and Experimental Pathology
Salt Lake City, UT

* Address correspondence to this author at:
ARUP Institute for Clinical and Experimental Pathology
500 Chipeta Way
Salt Lake City, UT 84108
Fax 801-584-5207
E-mail tanya.sandrock@aruplab.com

DOI: 10.1373/clinchem.2007.100396

Letters to the Editor

In Vitro Formation of Homocysteine in Whole Blood in the Presence of Anticoagulants

To the Editor:

We read with great interest the letter by Hübner et al. (1) on the stability of plasma sulfur-containing metabolites in the presence of anticoagulants (EDTA and citrate) and a homocysteine stabilizer. These authors observed time-dependent increases in plasma concentrations of homocysteine (Hcy) and similar increases in plasma S-adenosylhomocysteine (SAH) in EDTA- and citrate-containing whole blood after 24 h at room temperature. In discussing the mechanisms of these increases, they postulated that plasma Hcy is generated from SAH in erythrocytes by the SAH-hydrolase–catalyzed reaction, and Hcy and SAH leak into plasma from erythrocytes (1). They further noted that the inhibition of SAH hydrolase by citrate results in a smaller Hcy increase compared with EDTA, which they claimed does not inhibit this enzyme.

We recently reported the iron-dependent in vitro chemical formation of Hcy from methionine

\[ y = 1.0596x - 18.27 \]
\[ R = 0.96 \]

Fig. 1. Correlation between extracellular cAMP measured by RIA and intracellular cAMP measured by EFC-cAMP (n = 49) [SEE = 34.75; F = 513.15, P < 0.0001]. Assays were performed in triplicate; 130% of control is denoted by dashed lines. Normal is ≤109%; indeterminate is 110%–129%; a positive result is ≥130%.
(Met), SAH, and cystathionine, where EDTA or citrate (both 5 mmol/L) acted as a chelator (2). Based on these findings, we propose that a portion of the Hcy increases reported by Hübner et al. (1) results from the iron-dependent conversion of Met and SAH to Hcy. Furthermore, we showed that iron chelated by EDTA catalyzes the conversion of Met to Hcy more efficiently than iron chelated by citrate, and this should also be true for SAH (2). Although the inhibition of SAH hydrolase would prevent increases in plasma Hcy, this factor alone may not completely account for the differences in plasma Hcy increases of approximately 12 and 3 μmol/L in EDTA- and citrate-treated blood, respectively. One would expect that plasma SAH would be higher in the citrate-treated samples than EDTA-treated samples, if SAH hydrolase is inhibited by citrate. This is not true, because plasma SAH concentrations are 70–90 μmol/L at 24 h in samples containing EDTA or citrate. Plasma Hcy increases in whole blood treated with EDTA and 3-deazaadenosine (inhibitor of SAH hydrolase) after incubation at room temperature and 37 °C (3). Ubbink et al. (4) also reported that EDTA-treated blood produced a marked increase in plasma Hcy compared with Na-fluoride–treated blood. Na-fluoride may inhibit SAH production or SAH hydrolase; however, Na-fluoride is not an iron chelator and should not mobilize protein-bound iron. The amount of K₃ EDTA in evacuated tubes is about 4 mmol/L (1.8 g/L of blood), which is similar to the EDTA concentration (5 mmol/L) that we used in our in vitro study (2). Therefore, iron in both plasma and erythrocyte would be exposed to chelation, because EDTA concentration is higher than that of protein that binds iron. Both SAH and the larger pool of Met could react with iron chelated by EDTA, resulting in a larger-than-expected increase in plasma Hcy. Serum samples contain no iron chelator and a small amount of iron and should not show an increase in Hcy.

In addition to the mechanistic points presented here, Hübner et al. (1) reported that the 24-h in vitro increases in plasma Hcy concentrations in the presence of EDTA were only 0.1–9.5 μmol/L in patients with renal disease whose baseline plasma Hcy values were far higher than those of healthy subjects. In contrast, the increases in plasma Hcy in healthy subjects were much higher, range 8.7–23.6 μmol/L. These findings might be explained by the fact that blood iron concentrations in patients with renal disease are much lower than those in healthy subjects, since patients with chronic renal disease often have iron deficiency (5). The amount of iron that would be chelated by EDTA may be lower in these patients, resulting in a reduced production of Hcy from Met and SAH. It may be of interest to examine the correlation between 24-h Hcy increase and blood iron concentrations in these patients and healthy individuals.

Hübner et al. (1) noted that the proprietary material in Primavette™ affected the fluorescence polarization immunoassay of Hcy. Although we have not evaluated Primavette, it may be found to interfere with other laboratory analyses. We recommend that whole blood samples should be collected with citrate rather than EDTA as an anticoagulant and centrifuged as soon as possible. If this is not possible, whole blood samples should be kept refrigerated until plasma separation. Further studies are needed to elucidate the mechanism of Hcy increase.

**Grant/Funding Support:** None declared.

**References**


**Tsunenobu Tamura* Joseph E. Baggott**

Department of Nutrition Sciences
University of Alabama at Birmingham
Birmingham, AL

* Address correspondence to this author at:
Department of Nutrition Sciences
University of Alabama at Birmingham
Birmingham, AL 35294-3360
Fax 205-996-5775
E-mail tamurat@uab.edu

DOI: 10.1373/clinchem.2007.101642

**Estimation of Glomerular Filtration Rate by Use of Beta-Trace Protein**

**To the Editor:**

Because we recently introduced beta-trace protein (BTP) for estimating glomerular filtration rate (GFR) in patients after kidney transplantation (1), we read with interest the report by White and coworkers (2). These authors proposed a BTP-based equation to calculate GFR in renal transplant recipients. Because a separate second
cohort was not available in the reported study, a validation of the BTP-based formula was not performed. However, we were able to validate the suggested equation in 85 kidney transplant recipient patients who had participated in our study mentioned above.

The following equation of White and coworkers was applied:

\[
\text{GFR (mL/min/1.73m}^2\text{)} = 167.8 \times \text{BTP}^{-0.758} 
\times \text{creatinine}^{0.204} \times 0.871
\]

if the patient is female,

where BTP is given in milligrams per liter and creatinine is given in micromoles per liter. The computed results were compared to the reexpressed Modification of Diet in Renal Disease (MDRD) formula by determining correlation, bias, precision, and accuracy. As outlined in a previous report by White and coworkers, their creatinine is calibrated to the Cleveland Clinic laboratory (3). Thus, to provide comparability, we converted our isotope-dilution mass spectrometry-calibrated creatinine to the Cleveland Clinical Laboratory using an equation proposed by Levey et al. (4). GFR was determined by technetium-diethylentriamine pentaacetic acid clearance performed as a single-injection technique with a 2-point sampling approach at 1 and 3 h after intravenous injection, according to the method of Russell et al. (5). The mean GFR of the cohort was 38.6 (95% CI, 35.3–41.9) mL/min/1.73 m².

Correlation coefficients of the predicted GFRs by both formulae with the measured reference GFRs were high and did not differ significantly (BTP formula, \( r = 0.866\) vs MDRD equation, \( r = 0.863\)). Both equations overestimated measured GFR to a small but significant extent [mean GFR estimated by the BTP formula was 46.7 (95% CI, 43.0–50.4) mL/min/1.73 m² vs the mean GFR estimated by the MDRD equation, which was 45.3 (95% CI, 41.1–49.6) mL/min/1.73 m²; \( P < 0.001\) for both]. The bias of the MDRD equation was somewhat lower than that of the BTP formula (6.7 vs 8.1 mL/min/1.73 m², not significant). Precision (measured as SD of the mean difference between measured and estimated GFR) (6) tended to be better for the BTP formula (8.44 vs 10.03 mL/min/1.73 m²), but this difference in precision did not quite reach statistical significance (\( P = 0.058\)). The rates of predicted GFRs within 10%, 30%, and 50% of the measured GFR were comparable (BTP formula: 24.7%, 63.5%, and 84.7%, respectively, vs MDRD equation: 24.7%, 71.8%, 87.1%; not significant by McNemar test). Bland and Altman plots of the measured vs predicted GFRs of both equations are given in Fig. 1.

In conclusion, this external validation provides evidence that the performance of the BTP formula suggested by White and coworkers (2) is comparable to that of the reexpressed MDRD equation. Nevertheless, some difficulties were associated with the validation process and may have affected our results: (a) the validation cohort was relatively small, (b) although the same BTP determination technique was used, no international standardization was available, (c) differences in methods used for creatinine determination were corrected mathematically but no samples were sent to the Cleveland Clinical Laboratory for analysis, and (d) the cohorts appeared to
have different degrees of graft function [mean (SD) GFR 59 (23) in the patient cohort of White vs 38.6 (15.1) in our patient cohort].

Finally, an additional economic aspect must also be taken into account: BTP is considerably more expensive than creatinine. Thus, the suggested BTP-based equation for GFR calculation will gain public recognition only when its performance is demonstrated to be clearly superior to the MDRD equation. Further studies are needed to elucidate this issue more clearly.

Grant/Funding Support: None declared.

Financial Disclosures: None declared.

References


Uwe Po¨ge*
Thomas Gerhardt
Rainer P. Woitas

Department of Medicine
University of Bonn, Germany

* Address correspondence to this author at:
Department of Internal Medicine I
University of Bonn
Sigmund-Freud-Straße 25
D 53105 Bonn, Germany
Fax +49-228-2871-4952
E-mail dr.poeg@nephrologie-bonn.de

DOI: 10.1373/clinchem.2007.101840

Apolipoprotein A-II Is a Negative Risk Indicator for Cardiovascular and Total Mortality: Findings from the Ludwigshafen Risk and Cardiovascular Health Study

To the Editor:

Apolipoprotein A-II (apoA-II)1 was recently shown by Birjmohun and coworkers to be inversely associated with future risk for coronary artery disease in 912 cases and 1635 controls (1). This finding is important, because prior data have suggested that apoA-II has poor antiatherogenic properties and may even be proatherogenic. We now confirm and extend the antiatherogenic association of apoA-II to cardiac and total mortality on the basis of data collected after 8 years of follow-up in the Ludwigshafen Risk and Cardiovascular Health (LURIC) study, which is investigating patients who underwent elective coronary angiography (2).

Complete clinical and biochemical data from 3261 participants in the LURIC study are available. For these study participants, 752 deaths were recorded, and causes of death were obtained from official death certificates. Death from cardiac causes was recorded in 431 cases. Patients with (n = 2535) and without (n = 726) coronary artery disease presented with mean (SD) apoA-II concentrations of 0.44 (0.10) and 0.41 (0.09) g/L (P < 0.001), respectively. To examine the effect of apoA-II on cardiac and total mortality, we used Cox proportional hazards regression models to calculate hazard ratios (HRs). The data of the LURIC trial showed that apoA-II concentrations were negatively associated with cardiac death (HR 0.95, 95% CI 0.94–0.96; P < 0.001) and total mortality (HR 0.96, 95% CI 0.95–0.96; P < 0.001). For further risk assessment, quartile ranges of apoA-II concentrations measured in study participants without coronary artery disease were defined as <0.37 g/L (reference), 0.37–0.43 g/L, 0.43–0.50 g/L, and ≥0.50 g/L. The unadjusted HR for cardiac death of the fourth vs the reference quartile was 0.38 (95% CI 0.27–0.52; P < 0.001), and the HR per SD increase of apoA-II was 0.60 (95% CI 0.54–0.67; P for trend <0.001). However, in contrast to the findings of Birjmohun et al., we found that HRs of the fourth vs first quartile were 0.26 (95% CI 0.14–0.50; P < 0.001) for females (n = 990) and 0.45 (95% CI 0.31–0.64; P < 0.001) for males (n = 2271). For the total population, adjustment for age, sex, and body mass index; use of lipid-lowering drugs; presence of hypertension, diabetes, smoking, and/or alcohol consumption; and concentrations of triglycerides, LDL- and HDL cholesterol, and apoA-I resulted in HRs per SD increase of apoA-II of 0.66 (95% CI 0.57–0.78; P for trend <0.001) for cardiac death and 0.77 (95% CI 0.68–0.86; P for trend <0.001) for total mortality, respectively. If sensitive C-reactive protein was included, the HRs for the second and third quartile vs the 1st quartile remained significant only for cardiac death: 0.77 (95%
Letters to the Editor

CI 0.59–1.00; \( P = 0.047 \) and 0.69 (95% CI 0.50–0.96; \( P = 0.027 \)), respectively, with an HR per SD increase of apoA-II of 0.71 (95% CI 0.61–0.84; \( P \) for trend <0.001). After inclusion of N-terminal pro-B–type natriuretic peptide (3) into the model, however, the HRs for apoA-II were no longer found to be statistically significant.

Thus, the presented data from the LURIC trial clearly support the negative association of apoA-II with coronary artery disease and extend these findings to cardiac and total mortality. In contrast to the data reported by Birjmohun et al., our data indicate that this effect occurs in patients of both sexes, with an even more pronounced effect in females. When we included sensitive C-reactive protein in the analysis, the second and third quartile of apoA-II remained associated with cardiac death. However, further inclusion of N-terminal pro-B–type natriuretic peptide completely abolished the risk association of apoA-II. These findings support the hypothesis that apoA-II has a pathophysiological effect as an antiatherogenic apolipoprotein. Nevertheless, the usefulness of including apoA-II in risk stratification strategies is questionable because of the availability of more powerful risk markers, such as sensitive C-reactive protein and N-terminal pro-B–type natriuretic peptide.

Grant/Funding Support: None declared.
Financial Disclosures: None declared.
Acknowledgments: We thank the members of the LURIC study team who were involved in patient recruitment or sample and data handling and the laboratory staff at the Ludwigshafen General Hospital and the Universities of Freiburg and Ulm, Germany.

References

Karl Winkler2*
Michael M. Hoffmann2
Ursula Seelhorst3
Britta Wellnitz3
Bernhard O. Boehm4
Bernhard R. Winkelmann5
Winfried März6
Hubert Scharnagl7

2Department of Clinical Chemistry
University Medical Center Freiburg, Germany
3LURIC Study Nonprofit LLC
Freiburg, Germany
4Division of Endocrinology
Department of Medicine
University Hospital Ulm, Germany
5Cardiology Group
Frankfurt-Sachsenhausen, Germany
6Synlab Center of Laboratory Diagnostics
Heidelberg, Germany
7Department of Clinical Chemistry
University of Graz
Graz, Austria

* Address correspondence to this author at:
Department of Clinical Chemistry
University of Freiburg
Hugstetter Str. 55
79106 Freiburg, Germany
Fax +49 761 270 3444
E-mail karl.winkler@uniklinik-freiburg.de

DOI: 10.1373/clinchem.2008.103929

Instrument-Specific Matrix Effects of Calibration Materials in the LC-MS/MS Analysis of Tacrolimus

To the Editor:

LC-MS/MS can enable analyses on a reference method level in a routine laboratory, if stable isotope dilution internal standardization is applicable for an analyte. For many analytes, however, stable isotope-labeled internal standards are not available, and homologous compounds with a similar molecular structure must be used as an internal standard. The use of such homologs introduces a source of inaccuracy, because ion suppression (1, 2) and ion enhancement effects can have different impacts on the target analyte and the internal standard compound, respectively. This important pitfall of non-isotope dilution LC-MS/MS methods is illustrated by an observation we made in the context of monitoring the drug tacrolimus.

We have used 2 LC-MS/MS instruments for the quantification of tacrolimus, a Waters Quattro Ultima (instrument 1, installed 2003) and a Waters Micro (instrument 2, installed 2006). Similar methods were used on both systems, employing protein precipitation followed by on-line solid-phase extraction for sample preparation, as described previously (3). Ascomycin was used as the internal standard.

For logistical reasons, we implemented the same method on a third LC-MS/MS instrument (Waters Quattro LC, instrument 3, installed 2000). However, using instrument 3, we found the QC results to be unacceptably low, despite the use of identical preparations of commercial calibration materials as used for instrument 1 and instrument 2 (source A; Recipe, Munich, Germany; Lot 439). The QC samples were procured from a different commercial source.
(source B; Chromsystems, Munich, Germany).

Within distinct analytical series on the 3 instruments, the multiple-reaction–monitoring peak areas in the trace of ascomycin (internal calibrator) were similar in calibration samples, QC samples, and patient samples. All 3 instruments were tuned individually to obtain maximum ion yield for the ammonium adduct of tacrolimus, with single mass resolution. Identical mobile phases were used for all 3 systems.

To further investigate this observation, we procured calibration materials from source B and prepared a series of samples to be analyzed with the 3 instruments. This series included 6 calibrator samples from the 2 sources (A and B), and surplus whole blood from 20 random clinical samples submitted for tacrolimus quantification. After acquiring the primary data from the identical series on the 3 instruments, we performed 2 separate quantification runs on all 3 data systems. In the first quantification runs only the calibration materials from source A and in the second quantification runs only the calibration materials from source B were defined as calibrators. In this way, 6 different sets of results were generated for the 20 patient samples.

Use of the calibration materials from source A yielded mean tacrolimus concentration results from instrument 3 that were approximately 30% lower than the results from instrument 1 and instrument 2 (Table 1). In contrast, with the materials from source B excellent agreement was observed between all 3 instruments. Very close correlation between the 6 series was found for all comparisons, with no outliers ($r > 0.99$). Thus, the disagreement between the results from the 3 instruments that occurred with the use of materials from source A for calibration was a constant calibration inaccuracy.

After switching to calibration materials from source B, we obtained consistently acceptable internal and external QC results for instrument 3, and this system was used for clinical analyses.

Explanations remain speculative for this observed bias, dependent on instrument and calibration material, in the quantification of tacrolimus by LC-MS/MS. We suspect that the calibration material lot from source A contained compounds that interfered in an instrument-specific manner with the ionization of the target analyte tacrolimus as an ion enhancement, but not with the ionization of ascomycin (the internal standard).

Our observations highlight differential modulation of ionization efficacy of a target analyte and a respective internal standard compound by matrix constituents as an important potential source of error in LC-MS/MS methods in which the internal standard is a homologous compound instead of a stable isotope-labeled compound. Consequently, the important role of calibration material matrices in the accuracy of LC-MS/MS results must be considered in such methods. However, differential ion suppression or enhancement may also affect single individual samples of a series and may remain unrecognized. Not only such ionization-related effects, but also different stability of compounds, can introduce inaccuracy in non-isotope dilution LC-MS/MS methods (4). The development of stable isotope-labeled internal standards is thus of utmost importance for the application of LC-MS/MS in clinical laboratories. Our data furthermore underscore that the performance and interference characteristics of similar LC-MS/MS instruments can differ substantially, requiring instrument-specific method validation.

Grant/Funding Support: None declared.
Financial Disclosures: None declared.

References
Intracranial germ cell tumors account for 0.4%–3.4% of all brain tumors (1). These tumors can be divided into 2 groups, germinomas and nongerminomatous germ cell tumors. Germinomas, which are fairly treatable, arise from primordial germ cells that fail to migrate correctly in embryogenesis. Nongerminomatous germ cell tumors (including choriocarcinomas), which are more refractory to treatment, are differentiated tumors.

Measurement of human chorionic gonadotropin (hCG)1 is an important adjunct method in the diagnosis of germ cell tumors. At high concentrations hCG can be detected in serum, but measurement of hCG concentrations in cerebral spinal fluid (CSF) is a more sensitive and reliable indicator of tumor presence (1). Pure germinomas are associated with very low concentrations of hCG in both serum and CSF. A subset of nongerminomatous germ cell tumors contains syncytiotrophoblastic giant cells. These tumors are associated with moderately increased concentrations of hCG (<1000 IU/L) in the serum, CSF, or both, and the survival rate in patients suffering these tumors is poorer than that for patients with pure germinomas (2). In contrast, choriocarcinomas, another subset of nongerminomatous germ cell tumors, are associated with very high concentrations of hCG (>1000 IU/L) in both serum and CSF. Quantification of the hCG in CSF can be important in guiding treatment and monitoring response to treatment of these tumors (2).

Currently, all quantitative hCG assays in the US have been validated for use with serum only. Because matrix effects can influence test results when alternative sample types are used, the alternative sample type should be validated for the assay before clinical use. We have validated the Advia Centaur total hCG method, developed for use with serum and internally validated for use with urine (3), for use with CSF.

We performed the study with leftover samples collected for physician-ordered testing. Institutional review board approval was obtained for this study. We created a CSF pool by combining CSF samples from 50 patients. Chart review was performed to confirm that samples were from patients with no history of blood-brain barrier breakdown or central nervous system infection. Samples were accepted if they were clear and colorless. The limit of detection was evaluated by measuring hCG in the CSF pool 15 times. The mean hCG was 3.6 IU/L (range, 3.1–4.1 IU/L; SD, 0.4 IU/L). The minimum detection limit, calculated as the mean + 3SD, was determined to be 4.7 IU/L with a CV of 9.7%.

Recovery studies were performed by diluting hCG-positive serum from a patient with a nongerminomatous germ cell tumor into the pooled patient CSF. Because different isoforms of hCG are produced during pregnancy, cancer, and postmenopausal states, a patient sample was used in place of commercially available hCG to ensure that the assay was validated using the correct hCG isoform. Recovery studies were performed in duplicate on 2 different days. Results from 1 experiment are shown in Table 1. Recovery of added hCG was >100% at all concentrations in both experiments. Measurements of hCG in CSF were linear up to 400 IU/L [observed = (1.4 × expected) + 2.8; r² = 0.989].

The imprecision for twice-daily measurements over a 10-day period was evaluated by adding hCG-containing serum at 2 different concentrations to pooled patient CSF (total volume of serum added was <10%). Interassay imprecision (CV) was 4.6% at 153 IU/L and 2.6% at 510 IU/L.

It is important to note that most, perhaps all, hCG assays in

Table 1. Recovery of total β-hCG from CSF by the Advia Centaur total hCG assay.

<table>
<thead>
<tr>
<th>Expected, IU/L</th>
<th>Observed mean (range), IU/L (n = 2)</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>9 (9.1–9.4)</td>
<td>155</td>
</tr>
<tr>
<td>12</td>
<td>16 (15.5–15.8)</td>
<td>132</td>
</tr>
<tr>
<td>24</td>
<td>27 (27.3)</td>
<td>115</td>
</tr>
<tr>
<td>119</td>
<td>141 (139–143)</td>
<td>118</td>
</tr>
<tr>
<td>238</td>
<td>289 (288–290)</td>
<td>121</td>
</tr>
<tr>
<td>357</td>
<td>428 (427–429)</td>
<td>120</td>
</tr>
<tr>
<td>476</td>
<td>532 (528–536)</td>
<td>112</td>
</tr>
<tr>
<td>714</td>
<td>936 (923–949)</td>
<td>131</td>
</tr>
<tr>
<td>833</td>
<td>980 (978–980)</td>
<td>118</td>
</tr>
<tr>
<td>952</td>
<td>1090 (1007–1173)</td>
<td>115</td>
</tr>
</tbody>
</table>
the US are FDA approved for use as a marker for pregnancy, not as a marker for tumors. Nevertheless hCG assays are used to detect tumors. Various tumor types produce differing ratios of intact hCG to free $\alpha$-hCG and free $\beta$-hCG (4). In addition, different immunoassays preferentially recognize various forms of hCG (5). The Centaur assay does appear to preferentially recognize free $\beta$-hCG (5). This characteristic makes this particular immunoassay less than optimal for use in measuring hCG as a tumor marker and is the reason that the sample used for recovery assays was from a patient with a nongerminomatous germ cell tumor. Preferential free $\beta$-hCG recognition does not, however, account for the difference in expected vs observed concentrations that we observed, because the recovery was calculated on the basis of a nongerminomatous germ-cell tumor patient sample diluted into normal serum vs normal CSF. Therefore the difference in recovery is attributable to a matrix effect.

In conclusion, the Advia Centaur total hCG assay can be used to detect hCG in CSF with very good precision. Recovery of hCG added to CSF was $\geq 100\%$ at all concentrations tested, indicating a moderate matrix effect. This matrix effect should be taken into account when interpreting results. Any detectable hCG in CSF is abnormal. Very high CSF concentrations ($\geq 1000$ IU/L) suggest the presence of nongerminomatous germ cell tumors, which are usually refractory to treatment.

Grant/Funding Support: None declared.

Financial Disclosures: None declared.

References


Angela M. Ferguson
Bradley Ford
Ann M. Gronowski*

Department of Pathology and Immunology
Division of Laboratory Medicine
Washington University School of Medicine
Saint Louis, MO

* Address correspondence to this author at:
Department of Pathology and Immunology
Division of Laboratory Medicine
Washington University School of Medicine
660 South Euclid Avenue
Box 8118
Saint Louis, MO 63110
Fax 314-362-1461
E-mail gronowski@wustl.edu

DOE: 10.1373/clinchem.2008.106757

Immulite vs Scantibodies IRMA Plasma ACTH Assay

To the Editor:

The 2-site IRMA assay for adrenocorticotropic hormone (ACTH), as produced by Nichols Institute Diagnostics, was considered the gold standard in the US (1). This assay is no longer available. It was recently demonstrated that a manual IRMA ACTH assay produced by Scantibodies yielded results equivalent to those obtained with the Nichols Institute Diagnostics assay (2). We have observed that the results from the College of American Pathologists (CAP) Proficiency Survey samples for ACTH using the Scantibodies IRMA yielded higher results than those from the Immulite platform (Siemens), which is the assay used by the majority of clinical laboratories.

We analyzed 24 plasma samples, each from a different patient, selected to cover the critical range of plasma ACTH from 2.0–26.2 pmol/L (9–118 pg/mL). First, we evaluated the effect of freeze-thaw of the CAP proficiency samples by a large number of reporting laboratories, a procedure that is expressively proscribed by CAP, or that the Immulite platform yields lower plasma ACTH results than the Scantibodies IRMA.

We then measured plasma ACTH in split samples, each sample from a different patient, with the Scantibodies IRMA vs Immulite 2000 referred to ARUP Laboratories (Salt Lake City, UT). Fig. 1 shows the Bland-Altman plot of these findings (3). On average, the Immulite results were $-5.7\ pmol/L\ (-25.7\ pg/mL)$ (SD 4.0 pmol/L [18.1 pg/mL]; 95% CI 1.7 pmol/L [7.8 pg/mL]) compared to the Scantibodies result. This effect was not apparent in an

Clinical Chemistry 54:8 (2008) 1409
additional 5 samples from 5 different patients with high results ranging from 52–278 pmol/L (234–1253 pg/mL).

It is imperative that CAP Survey samples for ACTH be assayed immediately after reconstitution and without freezing, as instructed by CAP. When averaged over different patients, Siemens Immulite ACTH results are typically lower than those from the Scantibodies IRMA, and are probably typically lower than historical Nichols Institute Diagnostics values in the clinically significant critical range of plasma ACTH of 2.0–26.2 pmol/L (9–118 pg/mL).

Grant/Funding Support: None declared.

Financial Disclosures: Associated Regional and University Pathologists performed ACTH measurements on 29 samples for no charge.

Acknowledgments: The author thanks Barbara Jankowski and Peter Homar for their technical assistance.

References

Hershel Raff
Endocrine Research Laboratory
Aurora St. Luke’s Medical Center and Division of Endocrinology, Metabolism, and Clinical Nutrition
Medical College of Wisconsin
Milwaukee, WI

Address correspondence to the author at:
Endocrine Research Laboratory
Aurora St. Luke’s Medical Center 2801 W KK River Pky Suite 245
Milwaukee WI 53215
Fax 414-649-5747
E-mail hraff@mcw.edu

DOI: 10.1373/clinchem.2008.107987